

Embryotoxicity of the Alkylphenol Degradation Product 4-Nonylphenol to the Crustacean *Daphnia magna*

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Laboratory studies have suggested that some alkylphenols and pesticides elicit developmental toxicity to crustaceans. The purpose of the present study was to evaluate the possibility that the alkylphenol degradation product 4-nonylphenol is embryotoxic to the crustacean *Daphnia magna* through its known ability to interfere with the metabolic elimination of testosterone. Direct exposure of maternal daphnids to testosterone caused developmental abnormalities in neonates that consisted of partial arrest of early embryonic development and abnormalities in shell spine and first antennae development. Exposure of maternal daphnids to concentrations of 4-nonylphenol also produced developmental abnormalities though the profile of abnormalities was distinct from that observed throughout the testosterone concentration–response curve. Thus, 4-nonylphenol is a developmental toxicant in daphnids, but its toxicity is not consistent with that elicited by elevated testosterone accumulation. Further experiments demonstrated that testosterone was directly toxic to developing embryos, and the maternal organism can serve as the vector for this toxicity. In contrast, neither direct embryo exposure nor early maternal exposure to 4-nonylphenol elicited embryotoxicity consistent with that observed during continuous maternal and gestational exposure. Thus, 4-nonylphenol is not directly embryotoxic at these exposure levels, but rather toxicity is mediated by maternal influences during gestation. The threshold concentration for the occurrence of developmental abnormalities (~44 µg/L) indicates that typical environmental concentrations of 4-nonylphenol pose no imminent hazard with respect to developmental toxicity. However, these effects do occur at sufficiently low levels to warrant evaluation of the relative susceptibility of other crustacean species to this previously uncharacterized mode of toxicity. **Key words:** androgens, crustacean, *Daphnia magna*, development, embryo, invertebrate, 4-nonylphenol, steroids, testosterone, toxicity. *Environ Health Perspect* 108:1133–1138 (2000). [Online 2 November 2000]

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Arthropod (insects, crustaceans, and several minor phyla) endocrinology is dominated by the involvement of terpenoid, ecdysteroid, and peptide hormones (1). Some evidence suggests a role for vertebrate-type sex steroids (androgens, estrogens, progestogens) in regulating various reproductive processes in crustaceans (2). The paucity of the data argues that the observed effects of these steroids reflect the disruption of endocrine pathways that normally use other hormones rather than the stimulation of pathways that are specifically responsive to the administered hormone. Vertebrate-type sex steroids have been measured in crustaceans (2), and the question remains as to whether these compounds function in crustaceans as true signaling molecules (i.e., hormones), indirect regulators of physiological processes (i.e., modulators of enzyme activity), or inactive products of endogenous steroid hormones.

We have reported that the alkylphenol degradation product 4-nonylphenol alters the metabolic elimination of testosterone by the daphnid *Daphnia magna* (3). Testosterone is eliminated from this crustacean predominantly (> 90%) as the glucosylated derivative (4). Exposure of daphnids to 4-nonylphenol reduced the rate of elimination of testosterone as the glucosylated derivative while increasing

the rate of conversion of testosterone to various oxido-reduced derivatives (3). These derivatives (primarily 4-androstene-3,17-dione, 17β-hydroxy-5α-androstan-3-one, and 5α-androstan-3α/β,17β-diol) are relatively nonpolar and are preferentially retained by the daphnids compared to the polar conjugated derivatives (4). We refer to this disruption as “metabolic androgenization” because the elimination of inactive androgen is inhibited and the rate of testosterone conversion to derivatives that are variously androgenic in vertebrates is increased (3).

Recently, our laboratory has demonstrated that another chemical that elicits metabolic androgenization in daphnids, propiconazole, is also embryotoxic (5,6). This fungicide appears to interfere with the timing of embryo development resulting in deformities of the neonates, including underdeveloped antennae and a curved shell spine. Similar abnormalities were noted by Shurin and Dodson (7) during an evaluation of the toxicity of 4-nonylphenol to daphnids (*D. galeata mendotae*). These observations led us to hypothesize that metabolic androgenization is associated with embryotoxicity to daphnids, and this toxicity may be due to elevated testosterone levels within the maternal organisms. However, attempts to directly measure

changes in testosterone levels have been confounded by the small size of the organisms and the low levels of testosterone typically measured in crustaceans (8,9). If our hypothesis is correct, then direct exposure to testosterone should elicit embryotoxicity consistent with that observed with chemicals that elicit metabolic androgenization such as 4-nonylphenol and propiconazole.

The purpose of the present study was to evaluate both testosterone and 4-nonylphenol for embryotoxicity to daphnids. The embryotoxicity associated with each compound was assessed both qualitatively and quantitatively to discern whether the embryotoxicity of 4-nonylphenol might be due to elevated endogenous testosterone levels. Validation of our hypothesis that 4-nonylphenol elicits embryotoxicity by elevating maternal testosterone levels would provide evidence for a novel mechanism of embryotoxicity to these organisms that may be common among structurally diverse environmental chemicals.

Materials and Methods

Daphnids. Daphnids were cultured and experimentally maintained in deionized water reconstituted with 192 mg/L CaSO₄·H₂O, 192 mg/L NaHCO₃, 120 mg/L MgSO₄, 8.0 mg/L KCl, 1.0 mg/L selenium, and 1.0 mg/L vitamin B₁₂. Cultures were maintained at a density of 45 brood daphnids/L culture medium. Culture medium was renewed and offspring were discarded three times weekly. Brood daphnids were discarded after 3 weeks in culture and replaced with neonatal organisms. Cultured daphnids were fed twice daily with 1 mL (~4 mg dry weight) of Tetrafin fish food suspension (Pet International, Chesterfill, New South Wales, Australia) and 2 mL (1.4 × 10⁸ cells) of a suspension of the unicellular green algae, *Selenastrum capricornutum*. The algae were cultured in Bold's basal medium (10). The fish food suspension was prepared as described previously (11). Culture and experimental solutions were maintained at 20°C under a 16 hr photoperiod. These culture

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conditions maintained the daphnids in the parthenogenetic reproductive stage.

Embryotoxicity evaluations. The embryotoxicity of testosterone (Sigma, St. Louis, MO) and 4-nonylphenol (technical grade; Fluka Chemika, Ronkonkoma, NY) was evaluated using two exposure designs. In exposure design 1, we individually exposed daphnids (< 24 hr old) to concentrations of the test compounds in 50-mL beakers containing 40 mL culture medium. Each treatment consisted of 10 beakers, each containing a single daphnid. All treatments, including controls, contained the same amount (0.01% for testosterone experiments; 0.001% for 4-nonylphenol experiments) of vehicle solvent (absolute ethanol; AAPER, Shelbyville, KY). Daphnids were provided food twice daily (11), and test solutions were renewed three times weekly. Daphnids began releasing broods of parthenogenetically reproduced offspring on approximately day 7. We examined each beaker daily for released offspring. Offspring were counted, removed from the beakers, and examined microscopically to establish survival (heartbeat) and to identify any developmental abnormalities. We documented developmental abnormalities using a digital camera (Pixera Corporation, Los Gatos, CA) affixed to the microscope. Significant incidents of embryotoxicity were established following transformation of the percentages to their arcsin values, by Student's *t*-test when comparing a single treatment to the control or by analysis of variance and Dunnett's *t*-test when comparing multiple treatments to the control (12).

In exposure design 2, we generated concentration–response curves for embryotoxicity by exposing individual daphnids to each of at least 20 concentrations of the test compound, with each concentration being 90% of the next highest concentration. The exposure design was otherwise as described for exposure design 1. Concentration–response curves were generated by third-order polynomial regression using Origin software (Microcal Software Inc., Northampton, MA).

Direct embryo exposure. We conducted experiments to determine whether the embryotoxicity of the test compounds was the result of direct exposure of embryos to the compounds in the brood chambers of the maternal organisms. Gravid daphnids were selected from cultures and examined microscopically for the level of development of embryos in the brood chamber. Embryos that were in early development (i.e., stage 1, as described below) were removed by applying gentle pressure to the posterior region of the brood chamber with a dissecting needle. Extruded embryos were collected and pooled. Embryos were individually and randomly

assigned to wells of 96-well microtiter plates along with 200 μ L medium containing the desired concentration of test compound. Carrier solvent (ethanol) was present in all solutions at a concentration of 0.0005% (v/v). The number of embryos exposed to each treatment varied among experiments, depending on embryo availability, and is indicated for individual experiments in the “Results” section. Embryos were incubated at 20°C with a 16 hr photoperiod and were examined microscopically every 24 hr. We scored embryos for stage of development and recorded any abnormalities of development.

Developmental stages used to score the embryos were previously described and depicted (5) and are summarized as follows:

- Stage 1: cleavage, embryo is symmetrically enclosed within two embryonic membranes with no evidence of cellular differentiation
- Stage 2: gastrulation, cellular organization, and differentiation are evident; first embryonic membrane is ruptured as the embryo becomes asymmetrical; blastopore can be discerned
- Stage 3: early embryonic maturation; head capsule and second antennae are differentiated
- Stage 4: mid-embryonic maturation; eye becomes pigmented; second antennae remain confined by the second embryonic membrane
- Stage 5: late embryonic maturation; second embryonic membrane has ruptured, freeing the second antennae; shell spine remains curved along the anterior carapace edge
- Stage 6: fully developed neonate; setae evident on the second antennae, shell spine has fully extended from the carapace, and organism is freely swimming.

Treatment effects on the incidence of developmental abnormalities were statistically evaluated as described for exposure design 1.

Embryotoxicity after maternal exposure.

We conducted experiments to establish whether the embryotoxicity of the test compounds was a consequence of maternal exposure. Neonatal daphnids (< 24 hr old) were isolated from cultures and exposed to concentrations of the test compounds using exposure design 1. We collected embryos from the second or third broods produced by these organisms as described above.

Embryos were individually incubated in the wells of 96-well microtiter plates containing 200 μ L culture media with no test compound. The number of embryos exposed to each treatment varied among experiments, depending on embryo availability, and is indicated for individual experiments in the “Results” section. Embryos were incubated at 20°C under a 16 hr photoperiod and were examined microscopically every 24 hr. We scored embryos for stage of development and recorded any abnormalities of development. Treatment effects on the incidence of developmental abnormalities were statistically evaluated as described for exposure design 1.

Results

Embryotoxicity of testosterone. The embryotoxicity of testosterone was initially evaluated at an exposure concentration of 4.0 μ M. Preliminary experiments had indicated that 4.0 μ M testosterone would produce developmental abnormalities in offspring without adversely affecting overall performance of the maternal organisms. Exposure to 4.0 μ M testosterone had no effect on survival or total offspring production by maternal organisms but did produce developmental abnormalities among neonatal organisms (Table 1). Abnormalities associated with testosterone exposure were consistent, with both aberrations in late stages of embryo maturation (Figure 1B, Table 2) and early developmental arrest (Figure 1D, Table 2). Affected offspring were often released from the brood chamber with embryonic membranes still intact and often exhibited characteristics of multiple stages of development (Figure 1D), indicating perturbations in the timing of development.

Embryotoxicity of 4-Nonylphenol.

Exposure of daphnids for 3 weeks to 0.46 and 0.91 μ M 4-nonylphenol also resulted in a significantly high incidence of offspring with developmental abnormalities (Table 1). These exposure concentrations had no significant effect on the overall number of offspring produced per maternal daphnid. However, exposure to 0.91 μ M 4-nonylphenol was lethal to 50% of the exposed maternal organisms. Developmental abnormalities associated with 4-nonylphenol exposure were not consistent with the effects of testosterone on early embryonic development.

Table 1. Performance of daphnids exposed to concentrations of testosterone or 4-nonylphenol for 3 weeks.

Compound (concentration)	Parental survival (%)	Offspring/female (mean \pm SD)	Percent abnormal embryos (mean \pm SD)
Control	88	109 \pm 17	9.0 \pm 11
Testosterone (4.0 μ M)	89	93 \pm 36	28 \pm 16*
Control	100	76 \pm 17	8.1 \pm 3.1
4-Nonylphenol (0.46 μ M)	90	83 \pm 18	23 \pm 8.9*
4-Nonylphenol (0.91 μ M)	50*	77 \pm 14	83 \pm 16*

*Significant ($p < 0.05$) difference from the control daphnids.

Rather, developmental abnormalities consisted primarily of curved or unextended shell spines and underdeveloped first antennae (Table 2, Figure 2). The incidence of both of these abnormalities increased with increasing 4-nonylphenol exposure concentration. These observations suggest that both testosterone and 4-nonylphenol interfered with latter stages of embryonic development (i.e., stages 4–6), whereas only testosterone interfered with early stages of embryonic development (i.e., stages 1–4).

Concentration–response analyses.

Experiments were conducted to compare the concentration–response curves for the developmental toxicity of testosterone and 4-nonylphenol and to determine whether the differences in the types of developmental abnormalities observed with the two compounds may reflect differences in the distribution of developmental abnormalities at different exposure concentrations along the concentration–response curve. Both testosterone and 4-nonylphenol exhibited steep and essentially parallel concentration–response curves with respect to embryotoxicity (Figure 3). The similarity in concentration–response curves support the hypothesis that these compounds elicit developmental toxicity via the same mechanism. However, the distribution of developmental abnormalities was consistently different between the two compounds throughout the concentration–response curves. Testosterone

caused curved shell spines, underdeveloped antennae, and early developmental arrest throughout the range of effective concentrations (Figure 4). In contrast, 4-nonylphenol caused curved shell spines and underdeveloped first antennae throughout the effective concentration range, with no occurrence of developmental arrest (Figure 5). In addition, a significant number of neonates that exhibited abnormal shell spines as a consequence of 4-nonylphenol exposure had spines that curved dorsally. All abnormal shell spines resulting from testosterone exposure curved ventrally (Figure 1C). In addition, 4-nonylphenol increased fecundity of daphnids in a concentration-dependent fashion (Figure 6). Testosterone had no such effect but reduced fecundity at exposure concentration $> 7.6 \mu\text{M}$ (data not shown). Taken together, these results indicate that the developmental toxicity exhibited by testosterone and 4-nonylphenol to daphnids are mechanistically distinct.

Table 2. Percentage incidence (mean \pm SD) of specific developmental abnormalities associated with exposure to testosterone or 4-nonylphenol.

Compound (concentration)	Curved or unextended shell spine	Underdeveloped antennae	Early developmental arrest
Control	1.6 \pm 2.6	2.3 \pm 4.0	3.9 \pm 4.5
Testosterone (4.0 μM)	2.1 \pm 1.8	6.0 \pm 4.0*	16 \pm 13**
Control	6.5 \pm 4.0	4.2 \pm 1.7	0
4-Nonylphenol (0.46 μM)	19 \pm 6.9*	7.8 \pm 4.4	0
4-Nonylphenol (0.91 μM)	82 \pm 15*	55 \pm 15**	0

The sum of individual abnormalities is not expected to total percentage of abnormalities presented in Table 1 due to individual organisms exhibiting multiple abnormalities and the exclusion of dead neonates in which specific abnormalities could not be discerned due to decomposition.

*Significantly different from the control ($p < 0.05$). **Significantly different from the control ($p < 0.01$).

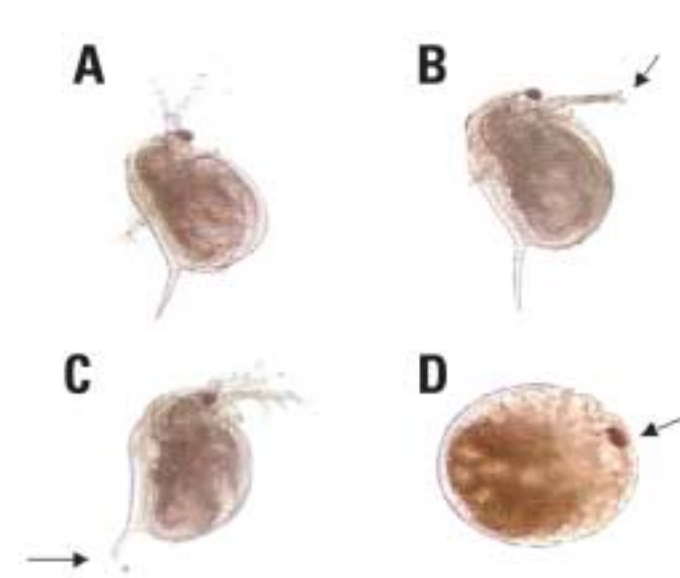


Figure 1. Abnormalities typically associated with neonatal daphnids produced during continuous exposure of maternal organisms to testosterone. (A) Normal neonatal daphnid (stage 6). (B) Neonatal daphnid with underdeveloped first antennae (arrow). (C) Neonatal daphnid with curved shell spine (arrow). (D) Neonatal daphnid that had undergone developmental arrest during early stages of embryo maturation; this embryo is of comparable age to the neonate shown in (A). A compound eye is evident (arrow) but, otherwise, the embryo appears to be in very early stages of development.

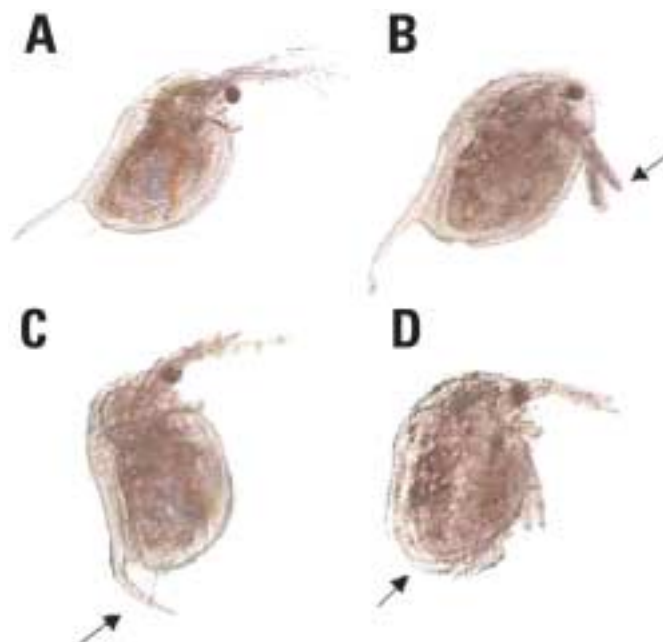


Figure 2. Abnormalities typically associated with neonatal daphnids produced during continuous exposure of maternal organisms to 4-nonylphenol. (A) Normal neonatal daphnid (stage 6). (B) Neonatal daphnid with underdeveloped first antennae (arrow). (C) Neonatal daphnid with curved shell spine (arrow). (D) Neonatal daphnid with unextended shell spine (arrow).

Similar experiments performed with 4-nonylphenol revealed that embryos incubated in solutions containing either 0.46 or 0.91 μM 4-nonylphenol developed normally with no significant incidence of embryotoxicity (Figure 8). Furthermore, no significant toxicity was observed among embryos derived from maternal organisms exposed to these concentrations of 4-nonylphenol that were allowed to develop in 4-nonylphenol-free medium (Figure 8). Thus, testosterone is directly embryotoxic to daphnid embryos, but embryotoxicity of 4-nonylphenol is elicited only as a result of exposure of gravid females (Figure 3).

Discussion

This study tested the hypothesis that testosterone is embryotoxic to daphnids and that chemicals which cause metabolic androgenization, such as 4-nonylphenol and propiconazole, are also embryotoxic by virtue of their ability to elevate endogenous testosterone levels. Results from this study clearly demonstrate that testosterone does interfere with normal embryo development in daphnids. Exposure of the mammalian fetus to

excess steroidal androgen can result in developmental aberrations (13). However, development and reproduction in daphnids differs significantly from those processes in mammals and a role for steroidal androgens in daphnid development is largely unknown. Under favorable environmental conditions, female daphnids produce diploid eggs that develop without fertilization. When stimulated by food deprivation or other environmental cues, the daphnids produce males and haploid eggs that must be fertilized to develop (14,15). The endogenous factors that regulate male production are not known; however, we have never observed the production of male offspring in response to testosterone exposure. Furthermore, the developmental abnormalities produced by testosterone exposure are not indicative of masculinization, but are more consistent with developmental arrest. Testosterone may elicit developmental toxicity to daphnids by interacting with a hormone receptor; however, this interaction likely reflects a disruptive interaction (i.e., binding antagonistically to an ecdysone receptor) rather than a constructive interaction (i.e., binding to a true

androgen receptor). Ecdysteroid antagonists have been reported to cause developmental abnormalities and reduce growth, longevity, and fecundity in insects (1). We have observed all of these effects with testosterone in daphnids during preliminary range-finding studies, supporting the possibility that testosterone elicits its embryotoxicity by acting as an ecdysone antagonist.

According to our working hypothesis, the previously described (4) metabolic androgenization caused by 4-nonylphenol results in testosterone-mediated embryotoxicity to daphnids. Indeed, results of this study confirm that 4-nonylphenol is embryotoxic to daphnids. However, detailed characterization revealed that the developmental toxicity of 4-nonylphenol is distinct from that associated with elevated testosterone. Specifically, a common toxicity associated with testosterone exposure, early developmental arrest (e.g., Figure 1D), was not associated with 4-nonylphenol exposure. Furthermore, dorsally curved shell spines occurred with 4-nonylphenol exposure but were not observed during testosterone exposure. These observations indicate that 4-nonylphenol does not elicit developmental toxicity by elevating endogenous testosterone levels.

4-Nonylphenol elicited the unexpected and unique effect of increasing overall fecundity of exposed daphnids. At this time, we cannot exclude the possibility that

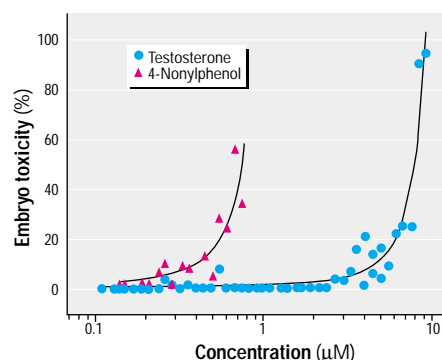


Figure 3. Concentration–response curves derived from exposure of daphnids to testosterone or 4-nonylphenol.

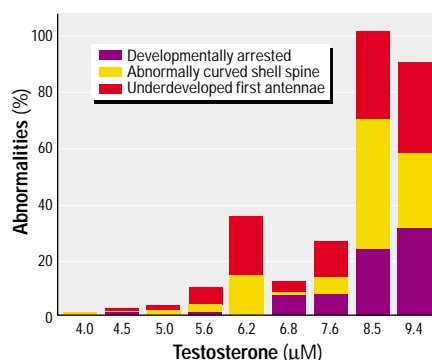


Figure 4. Composition of developmental abnormalities resulting from exposure of daphnids to concentrations of testosterone. No significant difference existed in the relative distribution of the three developmental abnormalities over the concentration–response curve.

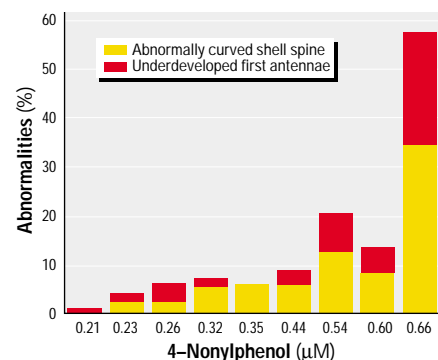


Figure 5. Composition of developmental abnormalities resulting from exposure of daphnids to concentrations of 4-nonylphenol. Developmental arrest of embryos did not occur at any exposure concentration. No significant difference existed in the relative distribution of the three developmental abnormalities over the concentration–response curve.

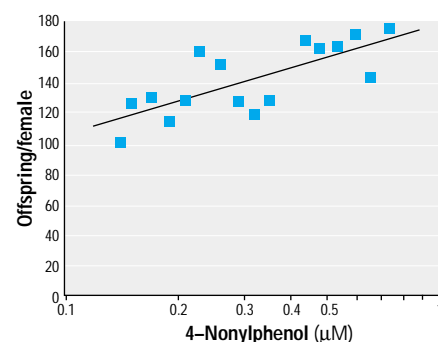


Figure 6. Total offspring produced by maternal daphnids during exposure to concentrations of 4-nonylphenol ($r = 0.72$, $p = 0.0015$).

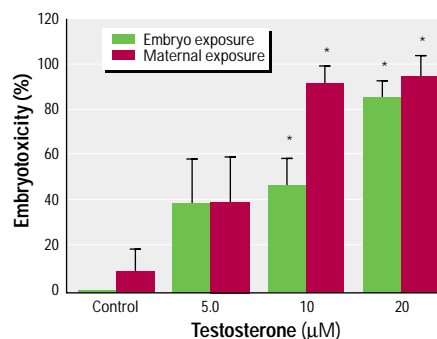


Figure 7. Toxicity resulting from direct embryo exposure or maternal exposure to concentrations of testosterone. Direct embryo exposures were performed with embryos removed from maternal organisms then cultured in solutions containing testosterone at the indicated concentrations. Results shown are the means \pm SDs from three separate experiments (10–20 embryos/treatment in experiment 1, 12–24 embryos/treatment in experiment 2, and 73 embryos/treatment in experiment 3). Maternal exposures were performed with embryos that were removed from maternal organisms exposed to the indicated concentrations of testosterone, and the embryos were cultured in solutions containing no testosterone. Results shown are the means and ranges from two separate experiments (12 embryos/treatment in experiment 1 and 24 embryos/treatment in experiment 2). *Significant ($p < 0.05$) difference from the respective control. No significant ($p < 0.05$) differences were discerned between paired data sets at all treatment levels.

4-nonylphenol stimulated the growth of algae provided as food and increased fecundity as a consequence of improved nutrition. However, there was no evidence of excess algae in any test solutions during these experiments. 4-Nonylphenol more likely had a direct stimulatory effect on egg production. Comber et al. (16) did not observe any increase in fecundity with 4-nonylphenol exposure. Shurin and Dodson (7) did report an approximately 2-fold increase in fecundity among daphnids exposed to 50 µg/L (0.23 µM) 4-nonylphenol compared to control daphnids and daphnids exposed to 10 µg/L (0.046 µM) 4-nonylphenol. The significance of this increase, however, was questionable because daphnids exposed to the carrier solvent (acetone) alone experienced increased fecundity. In the present study, carrier solvent (ethanol) was maintained at the same level among all 4-nonylphenol treatment levels, and the concentration–response was definitively analyzed between 31 and 163 µg/L (0.14–0.74 µM) 4-nonylphenol. These analyses unequivocally revealed the stimulatory effect of 4-nonylphenol on fecundity.

The increased egg production and increased proportion of developmentally compromised neonates observed with increasing 4-nonylphenol concentration opens the possibility that 4-nonylphenol stimulated egg production without increasing some critical developmental component provided to the eggs by the maternal organisms, such as ecdysteroids (17–19), essential fatty acids (20), or triglycerides (21). As a

result, more offspring were produced, but a significant percentage of the offspring were developmentally compromised. Alternatively, the metabolic effects of 4-nonylphenol characterized previously (3) using testosterone as a substrate may also be relevant to endogenous substrates resulting in perturbations in their provision to the newly produced eggs. For example, ecdysone of maternal origin is packaged into crustacean eggs largely as polar conjugates (18). The inhibition of the production of polar conjugates of ecdysone, as observed with testosterone (3), by 4-nonylphenol could limit the amount of ecdysone provided to the embryo with adverse developmental consequences. The observation that 4-nonylphenol was not directly embryotoxic to daphnid embryos but requires exposure of the gravid females during embryo development supports the hypothesis that 4-nonylphenol elicits embryotoxicity by interfering with the maternal provision of some constituents critical to normal embryo development.

The embryotoxicity of the fungicide propiconazole, as we have reported previously (5), closely resembles that of testosterone, as reported in the present study. Like testosterone, the developmental effects of propiconazole to daphnids consisted of underdeveloped antennae, ventrally curved shell spines, and early developmental arrest. Furthermore, propiconazole was a considerably more potent inhibitor of testosterone–glucose conjugation (6) as compared to 4-nonylphenol (3). Finally, maternal exposure to propiconazole was required to mimic the developmental toxicity observed during full life-cycle exposures. Taken together, these results suggest that propiconazole may elicit developmental toxicity by inhibiting the metabolic clearance of testosterone in maternal organisms, resulting in testosterone-induced toxicity to the embryos.

The concentration–response analyses for the developmental toxicity of 4-nonylphenol to daphnids suggest a threshold concentration of approximately 0.20 µM (44 µg/L). This value compares favorably with previously reported values based on daphnid reproduction (4,16). When detected, environmental concentrations of 4-nonylphenol in surface waters of the United States have typically ranged from 0.1 to 1.0 µg/L (22,23). Worldwide, surface water concentrations as high as 55 µg/L have been reported (22). These levels suggest that no widespread, imminent hazard exists with respect to the embryotoxicity of 4-nonylphenol to daphnids. They do substantiate the need to evaluate the extent to which other crustacean species are susceptible to this mode of toxicity and to establish the susceptibility of other crustaceans relative to daphnids.

In summary, results from this study demonstrate that daphnid embryos are susceptible to the toxicity of some environmental chemicals. The toxicity may be due to elevated steroidal androgen levels with chemicals that are potent inhibitors of the metabolic elimination of androgens (i.e., propiconazole). For other chemicals (i.e., 4-nonylphenol), the mechanism of embryotoxicity is distinct from that associated with testosterone.

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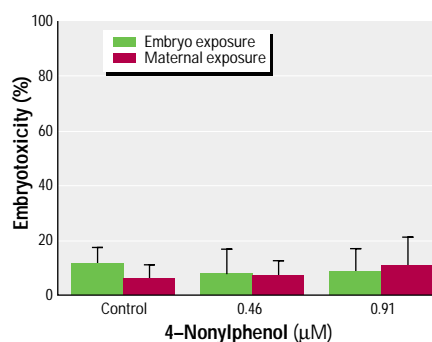


Figure 8. Toxicity resulting from direct embryo exposure or maternal exposure to concentrations of 4-nonylphenol. Direct embryo exposures were performed with embryos removed from maternal organisms then cultured in solutions containing 4-nonylphenol at the indicated concentrations. Results shown are the means \pm SD from three separate experiments (20–35 embryos/treatment). Maternal exposures were performed with embryos that were removed from maternal organisms exposed to the indicated concentrations of 4-nonylphenol, and the embryos were cultured in solutions containing no 4-nonylphenol. Results shown are the means \pm SDs from four separate experiments (24–36 embryos/treatment in experiment 1, and 70–112 embryos/treatment in experiments 2–4).

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